REMARKS

Prosecution

Applicant respectfully requests reconsideration of the outstanding rejections set forth in the Office Action mailed on January 9, 2006 in view of Applicant's instant claim amendments and the following Remarks.

Specification Amendments

Applicant respectfully requests entry of the above amendment to the title and submits that the above amendment does not constitute new matter. Support for the amendment to the title can be found throughout the specification and in the claims as originally filed.

Claim Amendments

Upon entry of the foregoing amendment, claims 1-50 are pending in the application. Claims 45-50 have been added. Claims 5, 13, 23, 27, 28, 33, 36-39, and 42 have been amended. The subject matter of claim 42 has been partially reiterated as newly added claims 45-46. Applicant respectfully requests entry of these claim amendments and new claims. Applicant respectfully submits that the claim amendments and new claims do not constitute new matter.

Support for the claim amendments can be found, *inter alia*, throughout the specification and in the claims as originally filed, and can be found, for example, in the specification at [076]; [090]-[092]; [181]-[203]; [287]-[293]; [414]; and [432]-[439].

Restriction Requirement

Applicant maintains their traverse of the Restriction Requirement mailed on June 21, 2005 for the reasons as set forth in the Response to Restriction Requirement filed on September 29, 2005. Applicant respectfully requests that all claims be rejoined and examined. Applicant reserves the right to pursue subject matter of non-elected claims in continuation, continuation-in-part, and divisional applications pursuant to 35 U.S.C. §§ 120 and 121.

Priority

A certified copy of the priority document FR 01/05516 is included herein.

Applicant respectfully requests that the foreign priority claim be acknowledged.

Oath/Declaration

The Office Action objected to the Oath/Declaration.

An executed Oath/Declaration was filed on February 9, 2004. A copy is included herein.

Reconsideration and withdrawal of the objection is respectfully requested.

Specification

The Office Action objected to the Title.

Applicant has amended the title to be clearly indicative of the invention to which the claims are directed.

Reconsideration and withdrawal of the objection is respectfully requested.

Claim Objections

The Office Action objected to claim 42 because it contained subject matter not elected by Applicant.

Applicant respectfully traverses this objection as improper because Office Action did not cite any support for the objection. However, Applicant has amended claim 42 rendering this objection *moot*.

Reconsideration and withdrawal of the objection is respectfully requested.

Written Description Rejection under 35 U.S.C. § 112 ¶ 1

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 112 ¶ 1 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed had possession of the claimed invention. Applicant respectfully traverses this rejection.

The Office Action failed to meet the legal standard for establishing a written description rejection because the Office Action only identified the claim limitations at issue but did not establish a *prima facie* case for providing reasons why a person skilled in the art at the time the application was filed would not have recognized that the invention was in possession of the invention as claimed in view of the disclosure of the application as filed. M.P.E.P. § 2163.04.

The specification provides sufficient disclosure to demonstrate that the inventors were in possession of the claimed genus of interferonα–17 (IFNα–17) polypeptides which share at least 95% sequence homology with SEQ ID NO: 2 wherein said polypeptides comprises the G45R SNP and retains IFNα–17 activity.

The Office Action asserts that the claimed genus has no common structure or function. Applicant respectfully notes that the claims require the polypeptide to share at least 95% homology with SEQ. ID. NO: 2, comprise the G45R SNP and have anti-viral and anti-tumoral activity. Applicant notes that despite some differences in their amino acid sequence, all of the polypeptides belonging to the IFNα family share a common structure and common anti-proliferative, antiviral, and immunomodulatory functions. Moreover, a comparison of the amino acid sequences of twelve immature IFNα proteins (IFNα–1, IFNα–2, IFNα–4, IFNα–5, IFNα–6, IFNα–7, IFNα–8, IFNα–10, IFNα–14, IFNα–16, IFNα–17, and IFNα–21) using CLUSTALW shows a global identity of 48.15% and a global similarity (including conservative amino acid substitutions) of 80.43%. In view of the art that teaches a family of proteins having about 80% homology, common structures, common functions, and the disclosure of the specification, it has to be conduced that the inventor was in possession of the genus of polypeptides instantly claimed when the application was filed.

Reconsideration and withdrawal of the rejection is respectfully requested.

Enablement Rejection under 35 U.S.C. § 112 ¶ 1

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 112 ¶ 1 because the specification, while being enabled for *an interferonα–17 variant of SEQ ID NO: 2 with a G45R/G22R SNP of the wild type protein which has antiviral activity*, the disclosure does not reasonably provide enablement for *all variants of interferonα–17 contemplated and which have any and all interferonα–17 activities*. Applicant respectfully traverse this rejection.

The Office Action failed to meet the legal standard for establishing an enablement rejection because the Office Action did not establish a *prima facie* case that undue experimentation is required to make and use the claimed invention. M.P.E.P. § 2164.01.

Applicant respectfully notes that to comply with the enablement requirement of 35 U.S.C. § 112 ¶ 1, it is not necessary to, "enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect." CFMT, Inc.

v. Yieldup, Int'l Corp., 349 F.3d 1333, 1338, 68 USPQ2d 1940, 1944 (Fed. Cir. 2003). Here, the specification provides sufficient disclosure on how to make and use a genus of interferonα–17 polypeptides which share at least 95% sequence homology with SEQ ID NO: 2 wherein said polypeptides comprises the G45R SNP and retains IFNα–17 activity. The specification teaches the primary structure of the genus of polypeptides (sequence), the necessary SNPs, and the activity along with methods to screen polypeptides. Id. at [432]-[439].

The Office Action asserts that the specification does not provide the structural and functional requirements to teach one of skill in the art to make and/or use the full scope of the claimed sequences.

Applicant respectfully notes that the claims require the polypeptide to share at least 95% homology with SEQ. ID. NO: 2, comprising the G45R SNP, and exhibiting anti-viral and anti-tumoral activity. Applicant notes that despite some differences in their amino acid sequence, all of the polypeptides belonging to the IFNα family share a common structure and common anti-proliferative, antiviral, and immunomodulatory functions. Moreover, a comparison of the amino acid sequences of twelve immature IFNα proteins (IFNα–1, IFNα–2, IFNα–4, IFNα–5, IFNα–6, IFNα–7, IFNα–8, IFNα–10, IFNα–14, IFNα–16, IFNα–17, and IFNα–21) using CLUSTALW shows a global identity of 48.15% and a global similarity (including conservative amino acid substitutions) of 80.43%. In view of the art teaches a family of proteins having about 80% homology, common structures, common functions, and the disclosure of the specification, the person of ordinary skill in the art has ample information on the structure and function of IFNα–17 polypeptides to make and use the full scope of the claims.

In consideration of the Office Action's discussion of the "Wands Factors", In re Wands, the court held that if, "all of the methods needed to practice the invention were well known," it would not constitute undue experimentation to make and use the invention. 858 F.2d 731, 740, 8 USPQ 1400, 1406 (Fed. Cir. 1998). For instance, Hu *et al.* (August 1, 2001) "Human IFN-alpha protein engineering: the amino acid residues at positions 86 and 90 are important for antiproliferative activity." J Immunol. **167**(3): 1482-9 (**Appendix A**) teaches that interferon-α along with interferon-β, and interferon-ω constitute a protein family that all share high homology in all three levels of protein structure, primary (sequence), secondary (α-helices and β-pleated sheets), and tertiary (three-dimensional structure). Id. at 1482. All of these proteins are related members of a cytokine family and comprise five helices. Hu *et al.* uses methods known in the

art at the time the application was filed to make and use mutants of IFNα–17 and screen them for activity. Id. at 1483; Figures 2, 5, 6. This demonstrates that changing the sequence of the IFNα–17 polypeptide was well-within the ability of a person of ordinary skill in the art at the time the invention was made.

Therefore, a person of skill in the art has sufficient guidance from the specification in the form of the sequences, the SNP required for the invention, detailed activities and methods for testing for them and a person of skill in the art could make and screen sequence variants encompassed by the claims with nothing more than routine experimentation.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. § 112 ¶ 2

Rejection of claims 27, 28, and 42 as indefinite

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 112 ¶ 2 as indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicant respectfully traverses this rejection.

Applicant respectfully submits that the claims as previously presented were clear and unambiguous when read in light of the specification. However, Applicant has amended claims 27, 28, and 42 rendering this objection *moot*.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection of claims 27, 28, and 42 as being vague and indefinite

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 112 ¶ 2 as vague and indefinite in the recitation of the term "equivalent position". Applicant respectfully traverses this rejection.

Applicant respectfully submits that the term "equivalent position" in the claims as previously presented was clear and unambiguous when read in light of the specification. However, Applicant has amended claims 27, 28, and 42 rendering this objection *moot*.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection of claim 42 as vague and indefinite.

The Office Action rejected claim 42 under 35 U.S.C. § 112 ¶ 2 as vague and indefinite in the recitation of the term(s), "substantially the same biological activity as the mature and immature form". Applicant respectfully traverses this rejection.

Applicant respectfully submits that the term(s) "substantially the same biological activity as the mature and immature form" in the claims as previously presented were clear and unambiguous when read in light of the specification. However, Applicant has amended claim 42 rendering this objection *moot*.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 102

Rejection under 35 U.S.C. § 102(a) or (e)

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 102(a) or (e) as being anticipated by U.S. Patent No. 6,299,877 (2001) Chen *et al.* ("the '877 patent"). Applicant respectfully traverses this rejection.

The present invention of claims 27 and 42 is drawn to polypeptides which have at least 95% sequence identity with the amino acid sequence of SEQ ID NO: 2 comprising the G45R SNP and have anti-viral and anti-tumoral activity. The present invention of claim 28 is drawn to polypeptides which have at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 2 comprising the G45R SNP and have anti-viral and anti-tumoral activity.

Since "[a]nticipation under 35 U.S.C. § 102 requires the disclosure in a single piece of prior art of each and every limitation of a claimed invention." Electro Med. Sys. S.A. v. Cooper Life Sciences, 32 USPQ2d 1017, 1019 (Fed. Cir. 1994), Applicant respectfully submits that the '877 patent fails to meet the limitation of the instant claims because the '877 patent does not teach a sequence with the G45R SNP. Id. at SEQUENCE LISTING. Therefore the '877 patent fails to anticipate the invention of claims 27 and 42 because it fails to teach a polypeptide which has at least 95% sequence identity with the amino acid sequence of SEQ ID NO: 2 comprising the G45R SNP. The '877 patent also fails to anticipate the invention of claim 28 because it fails to teach a polypeptide which has at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 2 comprising the G45R SNP.

Reconsideration and withdrawal of the rejection is respectfully requested.

Rejection under 35 U.S.C. § 102(b)

The Office Action rejected claims 27, 28, and 42 under 35 U.S.C. § 102(b) as being anticipated by Lawn *et al.* (June 5, 1981) "DNA sequence of two closely linked human leukocyte interferon genes." Science **212**(4499): 1159-62 ("Lawn *et al.*"). Applicant respectfully traverses this rejection.

The present invention of claims 27 and 42 is drawn to polypeptides which have at least 95% sequence identity with the amino acid sequence of SEQ ID NO: 2 and comprise the G45R SNP and have anti-viral and anti-tumoral activity. The present invention of claim 28 is drawn to polypeptides which have at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 2 and comprise the G45R SNP and have anti-viral and anti-tumoral activity.

Since "[a]nticipation under 35 U.S.C. § 102 requires the disclosure in a single piece of prior art of each and every limitation of a claimed invention." Electro Med. Sys. S.A. v. Cooper Life Sciences, 32 USPQ2d 1017, 1019 (Fed. Cir. 1994), Applicant respectfully submits that Lawn *et al.* fails to meet the limitation of the instant claims because Lawn *et al.* does not teach a sequence with the G45R SNP. Id. Therefore Lawn *et al.* fails to anticipate the invention of claims 27 and 42 because it fails to teach a polypeptide which has at least 95% sequence identity with the amino acid sequence of SEQ ID NO: 2 and comprising the G45R SNP. Lawn *et al.* also fails to anticipate the invention of claim 28 because it fails to teach a polypeptide which has at least 99% sequence identity with the amino acid sequence of SEQ ID NO: 2 and comprising the G45R SNP.

Reconsideration and withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the foregoing Amendment and Reply to the Office Action of January 6, 2006, Applicant respectfully submits that claims 27, 28, and 42 are in condition for allowance, and such disposition is earnestly solicited. Further, Applicant respectfully requests rejoinder of claims 1-26, 29-40, and 43-44, and allowance of claims 1-47. Should the Examiner believe that any patentability issues remain after consideration of this Response, the Examiner is invited to contact the Applicant's undersigned representative to discuss and resolve such issues.

In the event that a variance exists between the amount tendered and that deemed necessary by the U.S. Patent and Trademark Office to enter and consider this Response or to maintain the present application pending, please credit or charge such variance to the undersigned's **Deposit Account No. 50-0206.**

Respectfully submitted,

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APPENDIX A

Hu *et al.* (August 1, 2001) "Human IFN-alpha protein engineering: the amino acid residues at positions 86 and 90 are important for antiproliferative activity." J Immunol. **167**(3): 1482-9

Human IFN-α Protein Engineering: The Amino Acid Residues at Positions 86 and 90 Are Important for Antiproliferative Activity[1]

Renqiu Hu,[2]* Joseph Bekisz,* Hana Schmeisser,* Peter McPhie,† and Kathryn Zoon*

Human IFN-α is a family of structurally related proteins that exhibit a wide range of antiproliferative activities. To understand the structural basis for these different antiproliferative activities, eight recombinant human IFN-α hybrids (HY) of α21a/α2c (HY-4, HY-5) and mutants (site-directed mutagenesis (SDM)-1, 2 and cassette mutagenesis (CM)-1, 2, 3, and 4) have been expressed, purified, and characterized. The data showed that the amino acid region 81–95 is important for antiproliferative activity. Site-directed mutagenesis and cassette mutagenesis studies showed that if serine (S) 86 and asparagine (N) 90 were replaced by tyrosine (Y), the antiproliferative activity was increased. We have also observed that if Y86 was replaced by isoleucine (I), the antiproliferative activity was comparable. However, if Y86 was replaced by aspartic acid (D), lysine (K), or alanine (A), the antiproliferative activity was substantially decreased. Our results indicate that Y and/or I at position 86 and Y at position 90 are very important in antiproliferative activity of human IFN-α. Circular dichroism spectra showed that the amino acid replacements at position 86 did not change the secondary structure. Thus the biological activity changes among those mutants do not appear to be due to conformational changes. The results also suggest that hydrophobic residue(s) at position 86 may be important for the interaction of the molecule with its receptor. The competitive binding data correlated with the antiproliferative activity. The N-terminal region of the molecule and the hydrophobic residues (including Y and I) on the C-helix region at positions 86 and/or 90 are important for binding and antiproliferative activities of human IFN-αs. *The Journal of Immunology,* 2001, 167: 1482–1489.

Interferon (IFN)-α, IFN-β, and IFN-ω (type 1 IFN) constitute one of the most complex families of proteins exhibiting high homology in primary, secondary, and tertiary structure. These proteins are related members of the cytokine family and are composed of five helices (1, 2). In general, the effects of these IFNs are believed to be mediated through interaction with a common IFN receptor comprised of at least two proteins, IFNAR1 (3) and IFNAR2 (4–6), and signal transduction to the nucleus through the Janus kinase 1/STAT1 and 2 pathways. However, these IFNs have small differences in the primary, secondary, and tertiary structures that may be responsible for a significant variety of biological actions (7).

There are a large number of reports on the structure-function relationship of these IFNs (7–14). To elucidate the mechanism by which IFNs bind to and activate their receptor, detailed structure-activity studies are required. Currently, the structures of several IFNs have been solved, such as murine IFN-β (15), human IFN-α2 (16, 17), human IFN-β (18), ovine IFN-τ (19), and human IFN-α consensus (20). Numerous mutational studies have revealed important functional residues on IFNs (12). However, many of these studies predate the determination of the crystal structures of these molecules. Hence, it was not possible to design mutations based on any knowledge of the location of the mutated residue in the three-dimensional structure of the IFN molecule, or of its involvement in intramolecular interactions that are required for maintaining structural integrity (13).

Understanding the structure and function of human IFN-αs and their receptors is the primary focus of our studies. Previously we have purified and characterized 22 IFN-α components from the human lymphoblastoid cell line, Namalwa, induced with Sendai virus (21). One of them, component o, was found to be noteworthy for its high antiproliferative activity and its poor ability to compete for the IFN-α2b binding site (22). Based on our partial amino acid sequence data, component o was indistinguishable from IFN-α21a. Therefore, IFN-α21a was cloned and expressed (23, 24). To determine the specific region(s) of IFN-α responsible for antiviral and antiproliferative activity, three IFN-α2c and IFN-α21a chimeras, hybrid (HY)[3]-1 (IFN-α21a(1–75)/α2c(76–165)), HY-2 (IFN-α21a(1–95)/α2c(96–165)), and HY-3 (IFN-α2c(1–95)/α21a(96–166)), were constructed based on the three-dimensional structures of human IFN-α and -β, and the biological activities of these human IFN-αs were determined (23). HY-3 exhibited antiproliferative activity greater than either of the parent IFN-αs.

The goal of this study was to identify the amino acid residues of IFN-α hybrids that are important for antiproliferative activity. HY-4 (IFN-α21a(1–75)/IFN-α2c(76–81)/IFN-α21a(82–95)/IFN-α2c(96–166)) and HY-5 (IFN-α21a(1–81)/IFN-α2c(82–166)) were constructed using PCR. In addition, two mutant IFN-αs, site-directed mutagenesis (SDM)-1 (HY-4(86S→Y)) and SDM-2 (HY-4(90N→Y)), were prepared by SDM. Four additional mutants were constructed using cassette mutagenesis (CM): CM-1

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Received for publication February 13, 2001. Accepted for publication May 21, 2001.

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[1] This work is covered under U.S Patent Application PCT/US 99/15284.

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[3] Abbreviations used in this paper: HY, hybrid; SDM, site-directed mutagenesis; CM, cassette mutagenesis.

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0022-1767/01/$02.00

(SDM-1(86Y→D)), CM-2 (SDM-1(86Y→I)), CM-3 (SDM-1(86Y→K)), and CM-4 (SDM-1(86Y→A)). The antiviral, antiproliferative receptor binding properties of IFN-α hybrids and mutants were analyzed and compared. We have found that the region 81–95 and specifically amino acid residues at positions 86 and 90 are important in the antiproliferative activity and receptor binding of human IFN-α. In addition, the amino-terminal amino acid sequence is also important for interaction with the IFN-α receptor proteins.

Materials and Methods

IFNs

Recombinant human IFN-α2b was obtained from Schering-Plough (Kenilworth, NJ) and has an antiviral specific activity of 2×10^8 IU/mg protein. IFN-α2c cDNA was obtained from Stratagene (La Jolla, CA). HY-2 (α21a (1–95)/α2c (96–166)) with a antiviral sp. act. of 3×10^8 IU/mg protein were expressed and purified from *Escherichia coli* strain JM109 as previously described (23). IFN-α2b and IFN-α21a were radiolabeled with ^{125}I-labeled Bolton-Hunter reagent (Amersham, Arlington Heights, IL) as previously described (22, 23).

Cells and cell culture

Human Daudi cells were obtained from Dr. P. Grimley (Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, MD). Cells were grown in suspension using RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FBS, 2 mM glutamine, and 0.2% gentamicin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All cultures were determined to be free of mycoplasma.

Construction of IFN-α hybrids and mutants

Fig. 1 shows the construction scheme of IFN-α hybrids and mutants. HY-4 (IFN-α21a(1–75)/IFN-α2c(76–81)/IFN-α21a(82–95)/IFN-α2c(96–166)) and HY-5 (IFN-α21a(1–81)/IFN-α2c(82–166)) cDNA were constructed using PCR (23, 25) as shown in Fig. 1. PCR primers for the construction of HY-4 and HY-5 include 1) 5′-GXT GCT TGG GAT GAG ACC CTC CTA-3′, 2) 5′-TAG GAG GGT CTC ATC CCA AGC AGC-3′, 3) 5′-CTA GAC AAA TTC TAC ACT GAA CTC TAC CAG-3′, 4) 5′-CTG GTA GAG TTC AGT GTA GAA TTT GTC TAG-3′, 5) 5′-TCC GGA TCC TGT GAT CTG CCT CAG ACC-3′, and 6) 5′-GAG CTC GCA TGC TCA TCA TTC CTT ACT TCT TAA ACT-3′.

Primers 1, 2, 3, and 4 were used as inside primers for HY-4 (1, sense; 2, antisense) and HY-5 (3, sense; 4, antisense). Primers 5 and 6 were used as outside primers for both HY-4 and HY-5. The *Bam*HI restriction site (bold bases) is on the 5′ outside primer 5, and the *Sph*I restriction site (bold bases) is on the 3′ outside primer 6. Plasmid DNA pQE-30/HY-2 was used as template for the first PCR for HY-4 and HY-5. Purified DNA fragments from the first PCRs were mixed as templates, and primers 5 and 6 were used for the secondary PCR. The amplified final chimeric cDNA constructs were digested with *Bam*HI and *Sph*I (both obtained from Life Technologies) and ligated into pQE30 expression vector (purchased from Qiagen, Chatsworth, CA). The plasmids were then transformed into *E. coli* strain JM109 cells (Stratagene). Competent cells were made as previously described (23). All new constructs were confirmed by DNA sequencing.

Site-directed mutagenesis

SDM-1 (HY-4(86S→Y)) and SDM-2 (HY-4(90N→Y)) were generated using SDM (26). Plasmid DNA pQE30/HY-4 was used as the template for mutants SDM-1 and SDM-2 construction by PCR. The outside primers are primers 5 and 6, and the two inside mutant primers for SDM-1 are primer 7 (5′-GAA AAA TTT TAC ACT GAA CTT-3′, sense) and primer 8 (5′-αAG TTC AGT GTA AAA TTT TTC-3′, antisense). The other two inside primers for SDM-2 are primer 9 (5′-αCT GAA CTT TAC CAG CAG CTG-3′, sense) and primer 10 (5′-CAG CTG CTG GTA AAG TTC AGT-3′, antisense). The underlined bases are modified bases that yield the mutants. The amplified cDNA constructs were digested with *Bam*HI and *Sph*I and ligated into pQE30 expression vector, then the plasmids were transformed into *E. coli* strain JM109 cells. All constructs were confirmed by DNA sequencing with an automated DNA sequencing system.

Cassette mutagenesis

We also used a modification of CM (semisaturation mutation) (25, 27, 28) to generate multiple mutations of codons at amino acid position 86 in the SDM-1 for CM-1 (SDM-1(86Y→D)), CM-2 (SDM-1(86Y→I)), CM-3 (SDM-1(86Y→K)), and CM-4 (SDM-1(86Y→A)). The two outside primers for all CM mutants are primers 5 and 6. The degenerate inside primers for codon 86 of the SDM-1 mutants are primer 11, 5′-GAA AAA TTT (A/G/T) (C/T/A)(T/A) ACT GAA CTT AAC-3′ (sense), and primer 12, 5′-GTT AAG TTC AGT (T/A)(A/G/T)(C/T/A) AAA TTT TTC-3′ (antisense). Plasmid DNA pQE30/SDM-1 was used as template. The amplified modified cDNA constructs were digested with *Bam*HI and *Sph*I and ligated into the expression vector pQE30. Mutants were identified by DNA sequencing with an automated DNA sequencing system.

Expression and purification

All plasmid DNAs of IFN-α hybrids and mutants were individually transformed into *E. coli* strain JM109. Bacteria were grown in super broth (Biofluids, Rockville, MD) containing 100 μg/ml ampicillin in a 37°C shaker incubator overnight. The cultures were diluted 1/50 and incubated

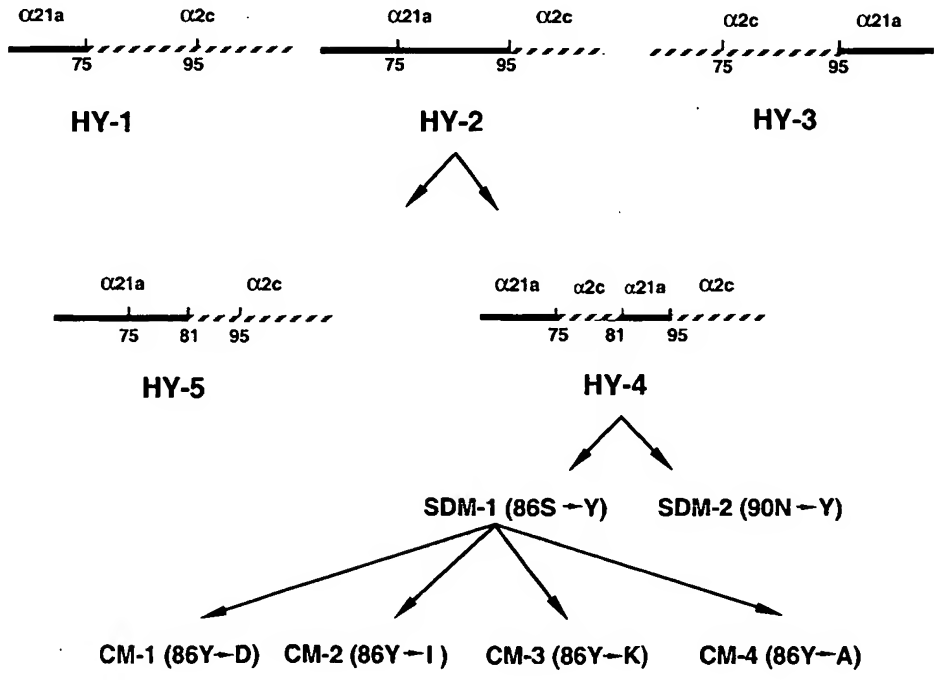

FIGURE 1. Construction scheme of human IFN-α hybrids and mutants.

at 37°C with shaking. Protein expression was induced by 1 mM isopropyl β-D-thiogalactoside. The bacteria were then incubated at 30°C for 4 h, after which cells were harvested by centrifugation and sonicated on ice (1-min bursts/1-min cooling/250 W) (23). IFN purification was performed by Ni-nitrilotriacetic acid agarose resin (Qiagen) (23, 29) and NK2 (a mAb against IFN-α) mAb affinity chromatography (21) as previously described (23).

Protein determination

Purified recombinant IFN protein concentrations were determined using the Coomassie Plus Protein Assay (Pierce, Rockford, IL). Purity of IFNs was assessed by SDS-PAGE (23) (data not shown).

Antiviral and antiproliferative assays

Antiviral and antiproliferative assays were performed as previously described respectively (21, 22). MDBK cells (American Type Culture Collection (ATCC), Manassas, VA) and Wish cells (ATCC) were used for the antiviral assay(s). All IFN units are expressed with reference to the National Institutes of Health Human Recombinant IFN-α2a standard Gxa01-901-535. Human Daudi cells were used for the antiproliferative assay.

Receptor binding assays

IFN-α receptor binding assays were performed as previously described on human Daudi cells (22).

Circular dichroism (CD) spectral analysis

CD spectra were measured at room temperature in a Jasco-J-715 spectropolarimeter at 25°C, using 1-mm-pathlength quartz cuvettes. Protein concentrations were 100 μg/ml in 5 mM sodium acetate buffer (pH 5.5), containing 0.1 M sodium perchlorate. Four scans were made between 260 and 185 nm, speed 50 nm/min, time constant equal to 1 s. Measured ellipticities (millidegrees) were converted into mean residue ellipticity. Secondary structure was determined from averaged spectral data using the CONTIN program (30).

Results

Expression, purification, and characterization of the IFN-α hybrids and mutants

Eight recombinant human IFN-α hybrids and mutants have been expressed and purified. To facilitate the purification process, an affinity tag consisting of six histidine residues was attached to the amino terminus of the recombinant IFN-αs. These His tags do not appear to interfere with the structure or function of the recombinant proteins (31).

Human HY-4 and HY-5, and mutants SDM-1, SDM-2, CM-1, CM-2, CM-3, and CM-4 were expressed in *E. coli* using a pQE30 expression system. Sequencing of the IFN-α hybrid and mutant cDNAs demonstrated that the mutated residues and positions agreed with the designed structure (Fig. 1). The expression levels of IFN hybrids and mutants were 0.5 mg/L (HY-4 and 5, and SDM-1 and 2) and 1 mg/L (CM-1, 2, 3, and 4). Initial purification of the IFNs on a Ni-nitrilotriacetic acid agarose column yielded partially purified protein, with antiviral specific activities ranging from 3 to 4.5 × 10^6 IU/mg protein on MDBK cells. These IFNs were further purified by NK2 mAb affinity chromatography (21). After the two purification steps, each IFN appeared as a single band with an apparent m.w. of 20,000 on reducing SDS-PAGE. The antiviral specific activities of the purified IFN-αs ranged from 2.0 to 4.6 × 10^8 IU/mg protein on MDBK cells. The antiviral specific activities of the hybrids and mutants were similar to each other on MDBK cells.

The ability of the IFN-α hybrids and mutants to inhibit the growth of Daudi cells was also examined. Some inhibition curves are shown in Fig. 2. The concentrations of these IFN-αs that inhibited Daudi cell growth by 50% are shown in Table I. Overall, HY-1 and 5, and mutants SDM-1, 2, and CM-2 have higher antiproliferative activities than HY-2, 4, and CM-1, 3, and 4. HY-3 exhibited the highest antiproliferative activity compared with the other hybrids and mutants on Daudi cells. In contrast, HY-2 has

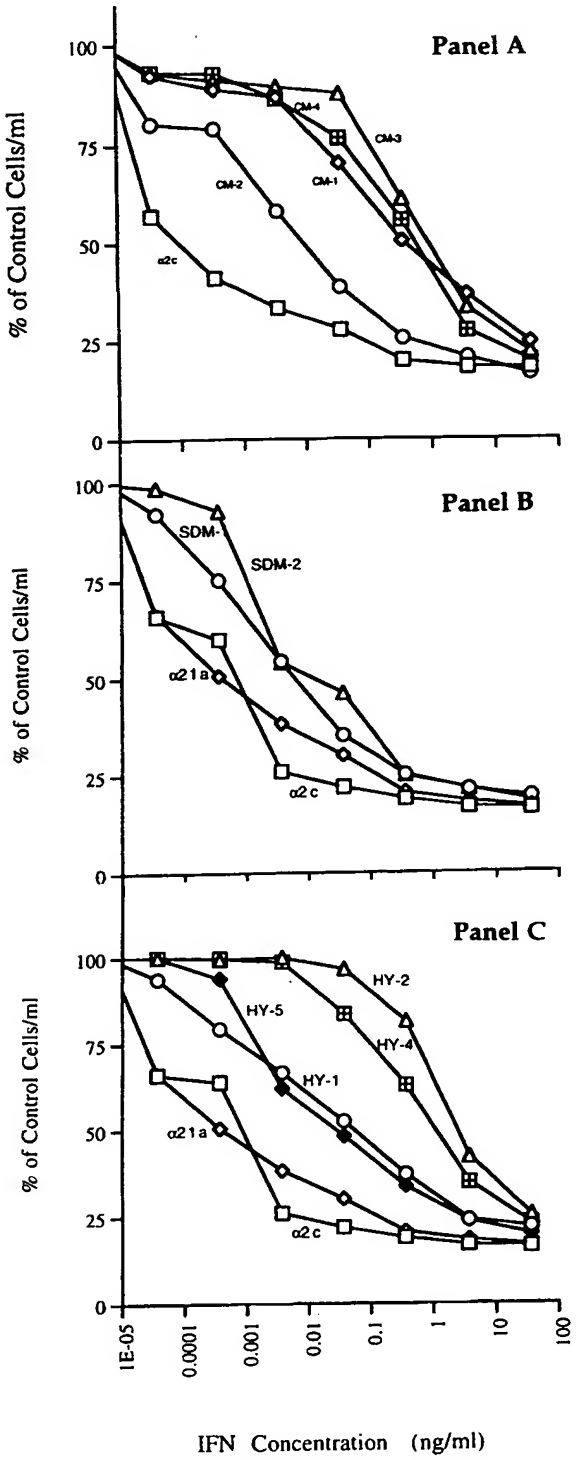

FIGURE 2. Inhibition of Daudi cell proliferation by human IFN-α hybrids and mutants. The number of Daudi cells present after incubation for 3 days at 37°C in the presence of the indicated concentrations of IFNs. The IFN-α2c (□) and IFN-α21a (◇) are used as controls. Results are expressed as a percentage of growth relative to a control cell culture. *A*, CM group. *B*, SDM group. *C*, HY group.

the lowest antiproliferative activity. SDM-1 displayed 300-fold higher antiproliferative activity compared with HY-2 on Daudi cells. Our earlier report (23) indicated that HY-1(IFN-α21a(1–75)/α2c(76–166)) had a 40-fold greater antiproliferative specific activity than HY-2 (IFN-α21a(1–95)/α2c(96–166)) on Daudi cells (23). Comparing the sequence of HY-1 and HY-2 there are seven

Table I. *Summary antiproliferative and binding activities of IFN-α hybrids and mutants*

IFN	Antiproliferative Activity on Daudi Cells (ng/ml)[a,b]	Binding Activity on Daudi Cells (ng/ml)[b,c]	
		^{125}I-labeled IFN-α2b	^{125}I-labeled IFN-α21a
HY-1	0.07 ± 0.015	600 ± 55	30 ± 14
HY-2	3.00 ± 0.80	2000 ± 153	250 ± 70
HY-4	1.5 ± 0.37	1000 ± 100	150 ± 50
HY-5	0.05 ± 0.02	1500 ± 104	75 ± 5
SDM-1	0.01 ± 0.002	400 ± 35	15 ± 5
SDM-2	0.02 ± 0.01	400 ± 20	50 ± 15
CM-1	0.6 ± 0.15	2000 ± 176	900 ± 100
CM-2	0.01 ± 0.005	400 ± 26	70 ± 6
CM-3	1.00 ± 0.20	800 ± 32	280 ± 30
CM-4	0.8 ± 0.20	1000 ± 153	420 ± 76
α21a	0.0008 ± 0.0002	2000 ± 208	60 ± 15
α2c	0.005 ± 0.0015	5 ± 2	250 ± 70

[a] Concentration that inhibits cell growth by 50%.
[b] Results taken from at least three individual assays.
[c] Concentration of IFN-α that inhibits binding of ^{125}I-labeled IFN-α2b (or ^{125}I-labeled IFN-α21) by 50%.

amino acid differences in the 75–95 region. Based on this data, we constructed HY-4 (IFN-α21a(1–75)/α2c(76–81)/α21a(82–95)/α2c(96–166)) and HY-5 (IFN-α21a(1–81)/α2c(82–166)). HY-4 has the 76–81 region of α2c and the same antiproliferative activity as HY-2 on Daudi cells. However, HY-5, which has the 82–95 region of α2c, has a 30-fold greater antiproliferative activity than HY-4 and 60-fold greater activity than HY-2 on Daudi cells. These data show that the amino acid region 81–95 is important for antiproliferative activity. Further comparing IFN-α21a and IFN-α2c there are only three amino acid differences in the 81–95 region (see Fig. 3). IFN-α2c has D, Y, and Y at positions 83, 86, and 90, respectively, and IFN-α21a has E, S, and N at these respective positions. Because the change from D to E is conservative, i.e., acidic amino acid residues are negatively charged, we constructed two mutants of HY-4 at positions 86 and 90, SDM-1 (HY-4(86S→Y)) and SDM-2 (HY-4(90N→Y)) by SDM. Fig. 2 and Table I show that if 86S was replaced by Y (SDM-1) or 90N was replaced by Y (SDM-2) the antiproliferative activity of HY-4 was increased similar to the level of HY-5. These data suggest that the tyrosines at positions 86 and 90 are important for antiproliferative activity. To determine whether only tyrosine at position 86 has this effect or whether other amino acid residues could substitute for tyrosine, amino acids that were either negatively or positively charged or that possessed long or short side chains were substituted using CM (32). Four mutants CM-1 (SDM-1(86Y→D), CM-2 (SDM-1(86Y→I)), CM-3 (SDM-1(86Y→K)), and CM-4 (SDM-1(86Y→A)) were selected. Tyrosine at position 86 was replaced with D, I, K, or A. We observed that I at position 86 (CM-2) had the same level of antiproliferative activity as SDM-1 (Table I). In contrast, replacement with D (CM-1), K (CM-3), or A (CM-4) decreased antiproliferative activity by 60-fold (CM-1), 100-fold (CM-3), and 80-fold (CM-4). These results suggest that tyrosine or isoleucine at position 86 is very important for the antiproliferative activity of IFN-α.

CD spectral analysis

Spectroscopic analysis was conducted on IFN-α mutants SDM-1, CM-2, and CM-4 to compare their secondary structure. Fig. 4 shows the far UV spectra of SDM-1, CM-2, and CM-4. These mutants all have very similar structures. Estimates of secondary structure were: 58–64% α helix, 9–40% β sheet for SDM-1; 63–71% α helix, 5–35% β sheet for CM-2; and 58–65% α helix, 9–42% β sheet for CM-4. These values were in the range of that described previously; human IFN-α has 66% α helix, 15% β sheet (14). Of interest, these results showed that the amino acid replacements at position 86 did not significantly change the secondary structure. Thus, the biological activity changes among those mutants do not appear to be due to major conformation changes.

Protein Sequencing of IFN-α2c, α21a, Interferon-α hybrids and mutants at amino acid region 75-95

	75						81					86				90				95
α 2c	A	A	W	D	E	T	L	L	D	K	F	Y	T	E	L	Y	Q	Q	L	N	D
α 21a	A	T	W	E	Q	S	L	L	E	K	F	S	T	E	L	N	Q	Q	L	N	D
HY-4	A	A	W	D	E	T	L	L	E	K	F	S	T	E	L	N	Q	Q	L	N	D
HY-5	A	T	W	E	Q	S	L	L	D	K	F	Y	T	E	L	Y	Q	Q	L	N	D
SDM-1	A	A	W	D	E	T	L	L	E	K	F	Y	T	E	L	N	Q	Q	L	N	D
SDM-2	A	A	W	D	E	T	L	L	E	K	F	S	T	E	L	Y	Q	Q	L	N	D
CM-1	A	A	W	D	E	T	L	L	E	K	F	D	T	E	L	N	Q	Q	L	N	D
CM-2	A	A	W	D	E	T	L	L	E	K	F	I	T	E	L	N	Q	Q	L	N	D
CM-3	A	A	W	D	E	T	L	L	E	K	F	K	T	E	L	N	Q	Q	L	N	D
CM-4	A	A	W	D	E	T	L	L	E	K	F	A	T	E	L	N	Q	Q	L	N	D

FIGURE 3. Amino acid sequence of IFN-α hybrids and mutants in region 75–95.

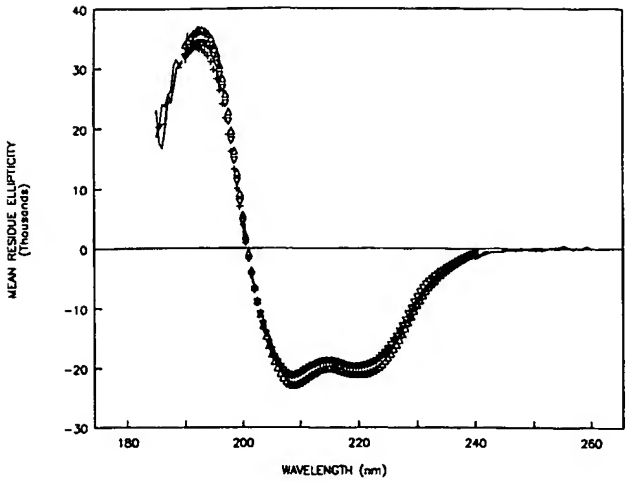

FIGURE 4. CD spectroscopy of IFN-α mutants. Protein concentration was 100 μg/ml in 5 mM sodium acetate, 0.1 M sodium perchlorate buffer, pH 5.5. Four scans were made between 260 and 185 nM. +, SDM-1; ◇, CM-2; ▽, CM-4.

Receptor binding properties

To examine the cell surface binding properties of IFN-α hybrids and mutants, competitive binding analysis was performed using ^{125}I-labeled IFN-α2b and ^{125}I-labeled IFN-α21a. Figs. 5 and 6 show examples of the ability of IFN-α21a, α2c, hybrids, and mutants to compete for IFN-α2b and IFN-α21a binding sites on Daudi cells. The concentrations of the IFN-αs, which inhibit binding by 50% on Daudi cells, are shown in Table I. IFN-α2c inhibited the binding of ^{125}I-labeled IFN-α2b to Daudi cells very effectively. The concentration of IFN-α2c, which inhibits binding by 50%, is 5.0 ± 2 ng/ml. In contrast, hybrids (HY-4 and 5) and mutants (SDM-1, 2, and CM-1, 2, 3, and 4) like IFN-α21a, which have the N-terminal part of IFN-α21a, poorly inhibited ^{125}I-labeled IFN-α2b binding to Daudi cells (Fig. 5 and Table I). The concentration of IFN-α21a, which inhibits binding of ^{125}I-labeled IFN-α2b by 50%, is 2000 ± 208 ng/ml on Daudi cells.

Fig. 6 shows the ability of IFN-α2c, α21a, hybrids, and mutants to compete for ^{125}I-labeled IFN-α21a binding sites on Daudi cells. HY-5 and mutants SDM-1, 2, and CM-2 compete very well and similarly with IFN-α21a for the ^{125}I-labeled IFN-α21a binding site. The concentration of IFN-α21a, which inhibits binding by 50%, is 60.0 ± 15 ng/ml. But HY-4, CM-1, 3, and 4 competed poorly for the ^{125}I-labeled IFN-α21a binding site on Daudi cells. CM-1 exhibits the lowest affinity for the IFN-α21a binding site (900 ± 100 ng/ml inhibits binding by 50%). Each of the IFN hybrids and mutants that competes well with ^{125}I-labeled IFN-α21a had tyrosine or isoleucine either at position 86 or 90 and had the N-terminal part of IFN-α21a or the original sequence of α21a in this region (86S, 90N). These binding results correlated with antiproliferative activity. Higher antiproliferative activity was observed for SDM-1 and 2, CM-2, and HY-5, whereas lower antiproliferative activity was seen for HY-4, CM-1, 3, and 4. Thus, these data suggest that the N-terminal region of the IFN-α molecule and amino acid residues at position 86 and 90 are important for the binding and antiproliferative activities of IFN-α.

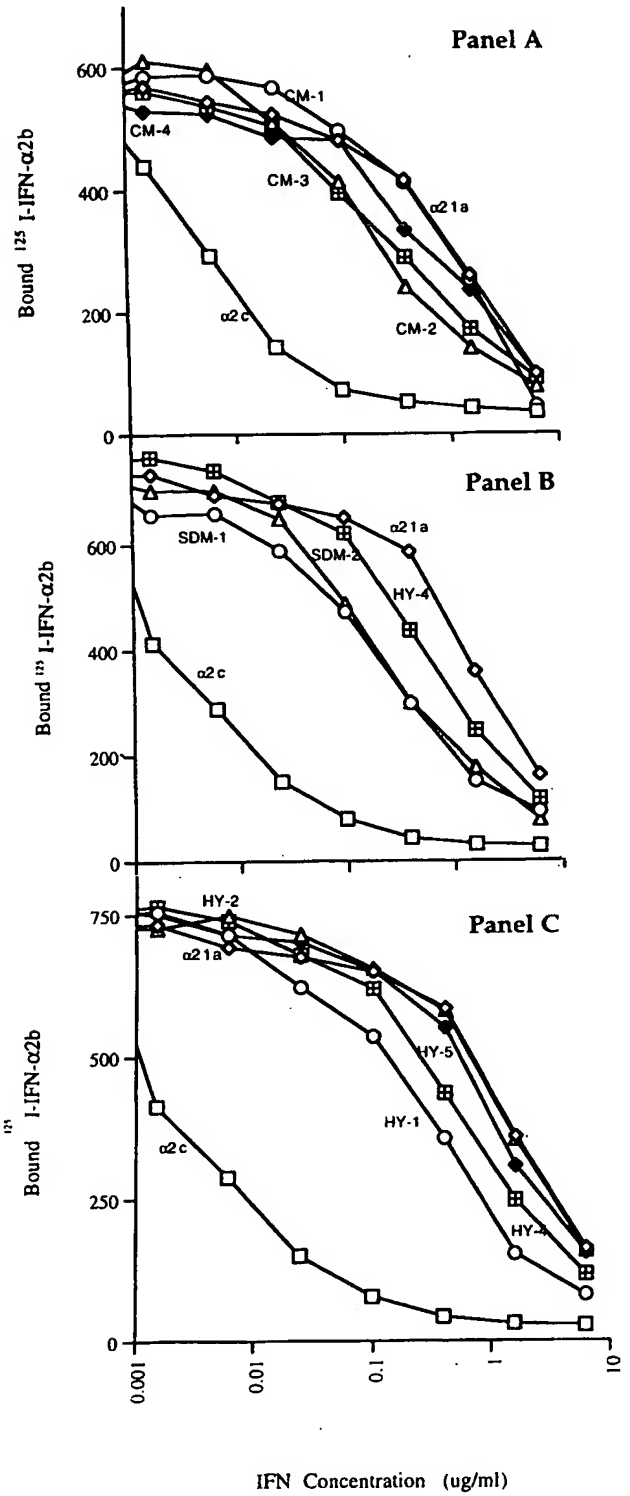

FIGURE 5. Competitive binding of IFN-α hybrids and mutants on Daudi cells using ^{125}I-labeled IFN-α2b. Unlabeled IFN-α2c (□) and IFN-α21a (◇) were used as controls, and IFN-α hybrids and mutants were used as competitors. The concentrations of ^{125}I-labeled IFN-α2b used in these experiments was 0.25 nM. *A*, CM group. *B*, SDM group. *C*, HY group.

Discussion

Comparisons of published structure and homology models of IFN-α and -β (7, 9, 31) show that the surface structures of these IFNs are likely to be involved in interaction with a specific cellular receptor, a step required to elicit a biological response in target cells. Several reports have proposed that two conservative amino acid regions 30–41 and 120–145 appear to constitute the basic framework of receptor binding site (14). The site in the N-terminal region would determine the binding to high-affinity receptors, and the site in the C-terminal region

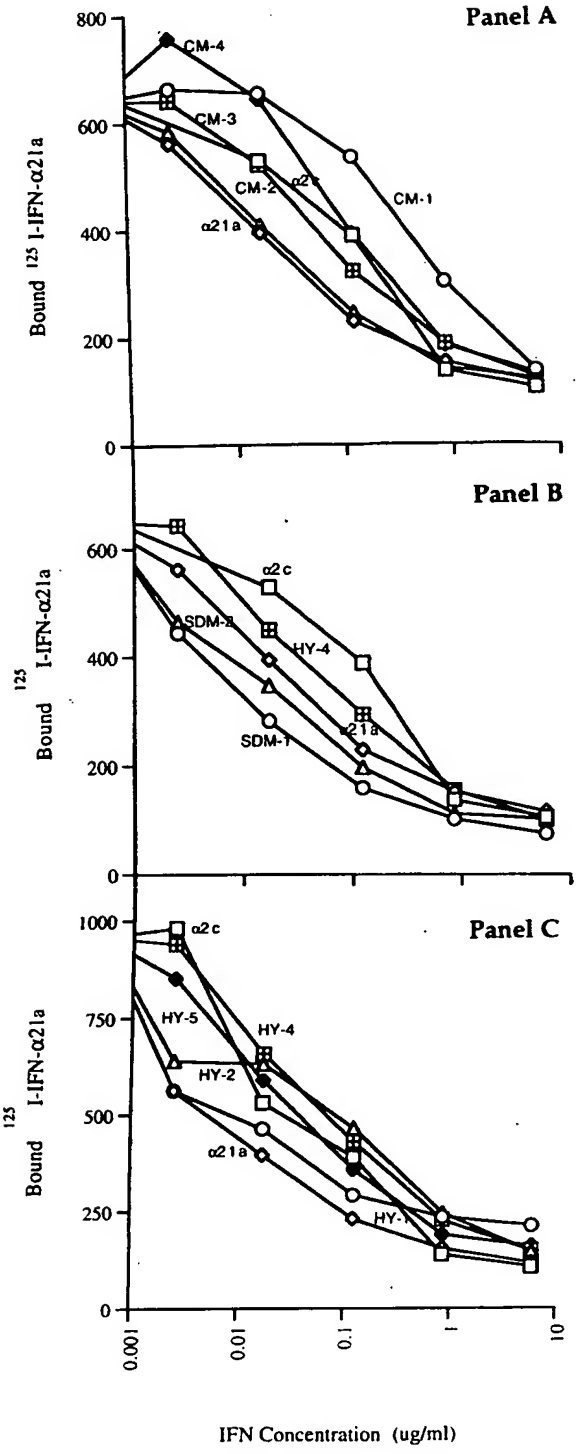

FIGURE 6. Competitive binding of IFN-α hybrids and mutants on Daudi cells using ^{125}I-labeled IFN-α21a. Unlabeled IFN-α2c (□) and IFN-α21a (◇) were used as controls, and IFN-α hybrids and mutants were used as competitors. The concentration of ^{125}I-labeled IFN-α21a used in these experiments was 0.25 nM. *A*, CM group. *B*, SDM group. *C*, HY group.

would influence low-affinity binding to cells (33, 34). Extensive evidence suggests that this receptor system is complex, possibly consisting of either multiple receptors or a multi-subunit receptor (22, 35). The amino acids in the 80–92 region (helix C) may be easily accessible to react with their appropriate receptors and thus may be important for biological activity. Dimarco et al. (8) conducted SDM in the C helix of the IFN molecule. They have proposed a model of IFN-receptor interaction in which these residues define a potential binding site. They have reported that mutated amino acids (at position 84, 86, 87, and 90) of helix C of IFN-α faces toward one of the subunits of the receptor and are part of one of two binding site of IFN-α. Korn et al. (20) and Fish (34) have proposed that there may be sufficient complexity in the type 1 IFN receptor (IFN-α and -β receptor) to account for the differential sensitivities between IFN-αs and IFN-β that may be associated with a residue difference in region 78–95. The IFN sequence 78–95 has been implicated as influencing species specificity among the murine and human IFN-αs and affecting the differential specificity of action between human IFN-αs and β. It is intriguing to speculate that these residue positions may constitute specific recognition sites associated with an accessory component of the IFN-α receptor. Earlier studies reported that the replacement of Ser^{86} of IFN-α4 with Cys increased the antiviral activity on murine cells by 10-fold, but did not affect the antiviral activity on human and bovine cells (36). Recent studies indicate that the high affinity binding site for human IFN-αβ is IFNAR2 (2, 13, 37). The binding face is formed by regions A, AB, and E of the IFNs. Based on the tertiary structure of IFN-αβ, it appears the helix C, which includes residues 86 and 90, is on the opposite face of the molecule and is more likely to bind to IFNAR1.

One prominent effect of the IFN-αs is their ability to inhibit cell growth, which has also been suggested to be of major importance in determining antitumor action. In our earlier studies the antiproliferative activity of several purified native and hybrid IFN-αs were compared (23). The data showed that HY-1 (IFN-α21a (1–75)/α2c(76–166)) has a higher antiproliferative activity than HY-2 (IFN-α21a(1–95)/α2c(96–166)) on Daudi and Wish cells, and that these two hybrids differ structurally by only 7 aa in the 75–95 region. To understand the structural basis for these different activities we used SDM and CM to create individual specific amino acid substitutions in this region. CM allows the manipulation of the IFN-α at position 86 with all possible amino acid substitutions to create several different mutants simultaneously. Our present studies show that HY-4 has the same antiproliferative activity as HY-2, but HY-5 like HY-1 has a higher antiproliferative activity than HY-4 and HY-2 (Fig. 2). The concentration of IFN that inhibits Daudi cell growth by 50% is 1.5 ng/ml for HY-4, and 0.05 ng/ml for HY-5 (Table I); HY-5 is 30-fold more active then HY-4. These data suggest that amino acid region 81–95 is important for antiproliferative activity. Further studies by SDM and CM show that tyrosines at positions 86 and 90 are important in the antiproliferative activity of human IFN-α. Likewise, substitution of 86Y with a hydrophobic amino acid residue, i.e., isoleucine, results in a similar level of antiproliferative activity and thus can replace Y at this position.

The members of the human IFN-α family, due to local differences in structure, sometimes show distinct properties. Even minute changes or differences in the primary sequences could be responsible for a significant variety of biological actions. Our results indicated that if the Y at position 86 or 90 is replaced, the biological activity also changes. The Y at position 86 seems to act as a spacer. The substitution of this residue with positively charged residue (K) like CM-3, negatively charged residue (D) like CM-1, or noncharged short chain (A) like CM-4, decreased the antiproliferative activity by 60-fold (CM-1), 100-fold (CM-3), and 80-fold (CM-4) compared with the original Y. However, substitution with isoleucine results in comparable antiproliferative activity to the tyrosine. The CD spectral analysis shows that the amino acid replacements at position 86 did not appear to change the secondary structure. The

Table II. *Correlation of structure and competitive binding activities*

IFN-αs	Activity	Structure		Concentration of IFN Required to Inhibit Binding of ^{125}I-labeled IFN by 50% (ng/ml)
		N-terminal	Y (I)[a]	
Compete with ^{125}I-labeled IFN-α2b				
HY-3	Competes best	α2b	Y	30
HY-1	Compete less	α21a	Y (I)	400–600
SDM-1,2				
CM-2				
HY-2,4	Compete least	α21a	—	800–2000
CM-1,3,4				
Compete with ^{125}I-labeled IFN-α21a				
HY-1,5	Compete best	α21a	Y (I)	15–75
SDM-1,2				
CM-2				
HY-2,4	Compete less	α21a	—	150–900
CM-1,3,4				

[a] Tyrosine (Y) or isoleucine (I) at position 86 or 90. −, No Y or I at position 86 or 90.

biological activity changes among those mutants cannot be attributed to conformation changes alone. It is possible that the amino acid residue at position 86 is involved in the interaction between IFN-α and its receptor. Our competitive binding data using ^{125}I-labeled IFN-α2b and ^{125}I-labeled IFN-α21a also provide evidence that demonstrated that the N-terminal region and residues at positions 86 and 90 are involved in IFN-α and receptor interaction. Table II shows the binding properties of IFN-α hybrids and mutants. If IFN hybrids and mutants have N-terminal part from IFN-α21a and Y (or I) at either position 86 or 90 (HY-1, HY-5, SDM-1, SDM-2, and CM-2), they compete best for the ^{125}I-IFN-α21a binding site on Daudi cells. However, HY-1, SDM-1, SDM-2, and CM-2 do not have the N-terminal of α2c, but they have Y or I at position 86 or 90, so they compete less for the ^{125}I-labeled IFN-α2b binding site. HY-2 and 4, CM-1, 3, and 4 have no Y at position 86 or 90 and have no N-terminal part from α2c; they compete least. It is possible that the differences in the competitive binding results obtained with the hybrids and mutants can be explained on the basis of the dual binding face human IFN-α and -β and their interaction with the IFNAR1 and IFNAR2 subunits. The fact that human IFN-α2c and IFN-α21a are poor competitors for one another might indicate that they possess discrete docking regions of interaction on the surface of IFNAR2. This is supported by our results in Figs. 5*B* and 6*B*. The SDM-1- and SDM-2-derived hybrids from HY-4 have the N-terminal 1–75 aa from α21a, but the majority of the remainder amino acid is α2c related in structure. These mutants compete strongly for α21a but not α2b. Thus, the A and AB domains appear to account for most of the binding specificity of these IFNs. In addition, SDM-1 and SDM-2 cause a significantly increasing affinity shift in the ability of HY-4 to compete with α21a binding (see Fig. 6*B*). These changes of Ser (SDM-1) and Asn (SDM-2) to Tyr make these molecules more like IFN-α2c. These data also suggest that this region may be involved in binding to IFNAR1 and that these mutants may be able to adopt a more favorable conformation.

The competitive binding experiments show that the IFN-αs with the IFN-α2c domain at the N terminus compete well with the ^{125}I-labeled IFN-α2b binding sites, and all hybrids and mutants with the IFN-α21a domain at the N terminus show reduced ability to compete with ^{125}I-labeled IFN-α2b binding sites. In contrast, the IFN-αs with α21a domain at N-terminal and that had Y at position 86 or 90 (SDM-1, 2, CM-2, and HY-5) compete well with ^{125}I-IFN-α21a binding sites. These data suggest that the N-terminal and the residues at positions 86 and 90 are involved in IFN-α receptor binding interaction. Cutrone et al. have recently defined a IFNα2 ligand binding site on IFNAR1. They reported that five alanine substitution of hydrophobic residues (132W, 139F, 141Y, 160Y, and 253W) on the receptor component, IFNAR1, could each decrease ligand binding by a minimum of 80%. These data suggest that the hydrophobic residues of the receptor are critical for their ligand interactions (38). The recent article of Piehler et al. (37) indicated that the IFNAR2 binding site overlaps the largest continuous hydrophobic patch on IFN-α2. Thus, hydrophobic interactions may play a significant role stabilizing this interaction with the charged residues contributing to the rapid association of the complex.

In conclusion, our data demonstrate that amino acid region 81–95 is important for antiproliferative activity, and that the tyrosine or the hydrophobic amino acid, isoleucine, at positions 86 and 90 are important for antiproliferative activity. Finally, positions 86 and/or 90 may be involved in the interaction of the IFN molecule with its receptor.

Acknowledgments

We thank Joan C. Enterline for performance of the antiviral assay, Dr. Ke-jian Lei for valuable discussions, and Drs. Blair Fraser and Raj Puri for review of the manuscript. Hana Schmeisser was supported in part by an appointment to the research participation program at Center for Biologics Evaluation and Research by Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

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